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Opinion

Engineering PGPMOs through Gene Editing and Systems Biology: A Solution for Phytoremediation?

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In light of extensive urbanization and deforestation, toxic wastes are being released into the atmosphere, causing increased air and soil pollution. Conventional methods of soil remediation are time consuming and labor and cost intensive, rendering them uneconomical to maintain sustainable agriculture. One solution is to use natural resources like plants and microbes for phytoremediation. A thorough systemic knowledge of plant–microbe interactions will allow the use of gene editing and gene manipulation techniques to increase the efficiency of plants in phytoremediation. This Opinion article focuses on gene editing techniques used in plants and microbes for phytoremediation and also emphasizes their effectiveness, advancement, and future implications for sustainable and environmentally friendly agriculture.

Highlights

Extensive urbanization and deforestation have increased soil toxicity, which has significantly affected the quest for sustainable agriculture.

Phytoremediation through plant–microbe interactions shows great promise for the removal of toxic elements from the rhizosphere, ameliorating the effect of heavy metals and strengthening nutrient uptake and increasing the bioavailability of metals as well.

To optimize the phytoremediation potential, an integrative systems biology platform could analyze a combined omics dataset to help identify candidate genes involved in the signaling network of plant–microbe interactions.

With a thorough understanding of gene functions, target genes could be modified using newly developed gene editing techniques, towards the development of interacting plants and microbes with enhanced phytoremediation properties for a greener future.

Towards More Sustainable Agriculture

The ever-increasing world population has led to mass deforestation to accommodate human growth and development, but the soil has slowly degraded in the process. Soil plays a primary role in sustaining life on Earth by supporting agriculture and controlling ecological balance through intricate regulation of biogeochemical and water recycling and, most importantly, by maintaining biodiversity [1,2]. Imprudent and constant use of pesticides and new generations of organic pollutants like polychlorinated naphthalenes (PCNs) and perfluorooctanoic acid (PFOA) by humans causes soil damage and imbalance in the ecosystem. The demand for more arable land for agriculture and habitation will increase, as will the use of pesticides and organic soil contaminants. Therefore, it is crucial to develop suitable agrobiotechnological approaches to judiciously use soil resources and help decontaminate the soil for extensive use in agriculture. One interesting alternative method to treat contamination is to use biological organisms for remediation [3]. Over the past few years, bioremediation or **phytoremediation** (see Glossary), typically referring to microbial or plant-based cleanup, has been used against a broad spectrum of hazardous compounds. In addition to soil cleanup, phytoremediation provides other advantages, like mitigation of soil erosion and control of solubilized contaminants by hydraulic activity. Growing plant species in contaminated soil provides several advantages, including sequestration of carbon, production of biomass or biofuel, and maintenance of biodiversity. However, plant-based phytoremediation is limited by several environmental extremes, such as the toxicity of pollutants and the influence of various environmental factors, such as soil texture, soil pH, vegetation reduction, and rhizosphere diversity. How plants and microbes cope with these adversities will be the determining factor in the establishment of phytoremediation methods as well-established strategies for sustainable agriculture in the future.

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Dissection of the mechanism of interactions between plants and plant growth-promoting microorganisms (PGPMOs) that contribute to a successful phytoremediation mechanism benefits from systematic design and the incorporation of principles of engineering. One such principle is the design–build–test–learn (DBTL) cycle – a pipeline that can be used recursively to develop a study design that can meet the desired specifics of the planned experiment [4,5]. Here we have utilized the DBTL cycle for a biological system (Figure 1). The cycle comprises inoculating plants with PGPMOs and observing physiological and phenotypic effects (design), generating datasets for both the plants and the PGPMOs using omics characterization (build), employing **systems biology** to integrate the datasets, identifying candidate genes by network biology, and ultimately evaluating their function by using gene editing (test and learn) to determine whether the design requires further improvement [6]. Thus, this Opinion article focuses on several gene manipulation techniques used in plants and microbes for phytoremediation methods. Also, it emphasizes the effectiveness of PGPMOs, including recent research advances and their future implications for a sustainable and environment-friendly agricultural system.

Mechanisms in Phytoremediation

Phytoremediation in plants comprises various mechanisms. Different mechanisms as defined by Tangahu and colleagues [7] and Pivetz [8] are described in Box 1, including phytoextraction, phytostabilization, phytodegradation, and phytovolatilization.

Role of Plant–Microbe Interaction in Shaping the Ecosystem

Symbiotic relationships of plants with rhizospheric organisms are known to contribute to the successful survival of plants under toxic and nutrient-limiting environments [2,9]. Although many research studies have been conducted, the prospect of using plant–microbe interactions for bioremediation remains murky, and optimizing the interaction between the plants and the microorganisms requires greater mechanistic understanding. In the plant symbiotic interaction, **microbiomes** are actively involved not only in altering host development and enhancing tolerance to diseases or abiotic stress but also in modulating the niche that the microorganisms occupy. Therefore, the 'hologenome' of the microbiomes can function as a buffer that can be easily manipulated according to environmental effects [10]. Recent research studies have identified the potential of genetic manipulation in plants and microbes to improve the interaction of plants with soil microorganisms and similar results have been obtained by applying

Box 1. Mechanisms of Phytoremediation

Phytoextraction occurs in plant roots, where contaminants are absorbed and translocated into various harvestable plant parts (e.g., shoots) that are converted into energy when burnt, and metal can be recycled from the ash [4]. This approach usually involves hyperaccumulators or plants that can accumulate 0.1% or higher of contaminants on a dry weight basis. This approach has been applied in Denmark using poplar and willow trees grown in polluted sites [6].

Phytostabilization utilizes plant species that can grow in contaminated soil that immobilize soil and groundwater contaminants through accumulation and absorption in the various tissues of the plants and adsorption into the roots or prevent migration in the soil by precipitation within the root zone or movement by deflation and erosion [4,5].

Phytodegradation refers to the degradation, uptake, and metabolism of contaminants from soil, sludges, sediments, and groundwater or surface water through enzymes that plants produce and release. Phytodegradation does not rely on rhizosphere-associated microorganisms.

Phytovolatilization occurs when a plant absorbs a contaminant that is modified or converted into another form that the plant releases into the atmosphere. For example, phytovolatilization begins as growing trees and other plants take up water with contaminants. Some contaminants can pass through the trees or plants and then volatilize in low concentrations into the atmosphere.

Glossary

Ethylene effect: a phytohormone produced by plants through the breakdown of methionine, ethylene affects plants in a variety of ways and dependent on the age and the sensitivity of the plant to ethylene. Some of the obvious effects include fruit ripening, chlorophyll loss, shortening or bending of the stem, abscission, and abortion of plants. Ethylene-mediated root-growth inhibition occurs due to the regulation of auxin biosynthesis or translocation and distribution of auxin. In addition, it has been shown to have a regulatory effect on abiotic and biotic stresses.
Gene editing tools: provide precise and permanent genome modification.
Hyperaccumulators: plants that can grow in soils contaminated with metals, which are absorbed by the plants' roots and translocated in various parts of their tissues.
Metabolomics: a technique that is focused on identifying specific, or a definite group of, metabolites in a cell type in response to some change or condition.
Metaorganism: a conglomeration of different organisms that are interacting in a biological network, as identified from a metagenome.
Microbiome: the collective community of microorganisms, possibly including archaea, bacteria, protists, fungi, and viruses, residing in plant niches.
Next-generation sequencing (NGS): high-throughput DNA sequencing techniques that are not Sanger based. The primary advantage of NGS over Sanger's method is that it removes the necessity for fragment cloning and can simultaneously sequence millions of DNA strands in parallel, yielding higher throughput.
Phytoremediation: remediation of selective contaminants from soil, sediment, ground water, surface water, sludges, or wastewater using the anatomy and physiological processes of plants.
Proteomics: a technique used to study the total or differentially expressed protein content of a living cell or tissues or of different organisms.
Systems biology: the study and understanding of interrelationships

exogenous chemicals [10–14]. Overexpression of *Escherichia coli* arsenate reductase (*arsC*) or γ-glutamylcysteine synthetase (*γ-ECS*) significantly increased tolerance to arsenate as observed through increased biomass and hyperaccumulation of arsenic in aboveground biomass [15]. Similar results of improved phytoremediation of mercury-contaminated soil have been observed in transgenic plants overexpressing bacterial mercury reductase genes [16]. Metagenomics, metatranscriptomics, and **metabolomics** have been identified as tools to identify novel genes for enhanced resource use by employing transgenic and designer plant technologies. A combination of new molecular techniques and genomics can elucidate rhizospheric interactions or plant–microbe interactions in the rhizosphere [7]. Similarly, a competition-driven model for the rhizosphere microbiome and a predictive framework for microbiome engineering [11] can be employed towards the enhancement of microbiome remediation attributes. Additionally, rather than genetically manipulation of plants, genetic manipulation of PGPMOs as biocontrol agents for various pathogens might be more useful in the long term. Several phenolic compounds and organic acids have shown potential in improving phytoremediation efficiency by acting as chelating agents. Thus, in the future it seems likely that comprehensive screening and isolation of signaling molecules from the root will enable modifications to the rhizospheric community for enhanced phytoremediation potential. In the context of the DBTL cycle, identifying the effect of plant inoculation with these beneficial microbes and knowing the associated phenotypic changes constitute the design section.

between biological components and the network of biological processes.
Transcriptomics: a technology that studies an organism's transcriptome by considering the sum of all of its RNA transcripts.

A Holistic Approach to Improve Plant–Microbe Interactions for Phytoremediation

Omics Approaches

The response of plants to various environmental cues involves various routes from changes in gene expression (i.e., the transcriptome) to defense responses and the accumulation of protein products that can degrade pollutants or metabolites as protectants. The introduction of **next-generation sequencing (NGS)** technology coupled with mass spectrometry into the global field of science and technology has made a significant contribution to our understanding of plant–microbe interactions and the addressing of other issues related to soil remediation. Large-scale omics studies are routinely used to understand the cellular processes, genetic control, and signaling networks involved in plant responses to environmental stresses [17,18]. Figure 1 illustrates the integration of large-scale studies to understand how plants respond to inoculation with PGPMOs under heavy metal-polluted soil (Table 1).

Transcriptomics, Proteomics, and Metabolomics

Manipulation of gene expression in plants through genetic engineering for use in phytoremediation is focused on: (i) manipulating uptake systems and transporter genes carrying metals or metalloids; (ii) enhancing the production of ligands from metals and metalloids; and (iii) converting metals and metalloids to forms that are less toxic and volatile [19,20]. Transcriptome analysis has played a significant role in identifying candidate genes like *Yeast Cadmium Factor 1* (*YCF1*) and a plasma membrane channel protein (*NtCBP4*), which, when overexpressed in plants, resulted in tolerance to cadmium (Cd) or lead (Pb^{2+}) [21,22]. In addition, this technology has been fundamental in the identification of several metabolic pathways that are altered in response to stress, as was observed in a study conducted on the response of rice to mercury stress [11]. Holmes and colleagues [23] utilized transcriptome profiling to identify upregulated genes in *Geobacter uraniireducens* used in bioremediation of uranium-contaminated aquifers. A large-scale gene expression analysis showed that while growing on sediments a range of genes, including iron–sulfur cluster-binding proteins, many c-type cytochromes, and hydrogenases [23], accumulated in increased abundance. Similarly, a study conducted on various bacteria, such as *Rhodococcus*, *Comamonas*, *Ralstonia*, and *Burkholderia*, to analyze the

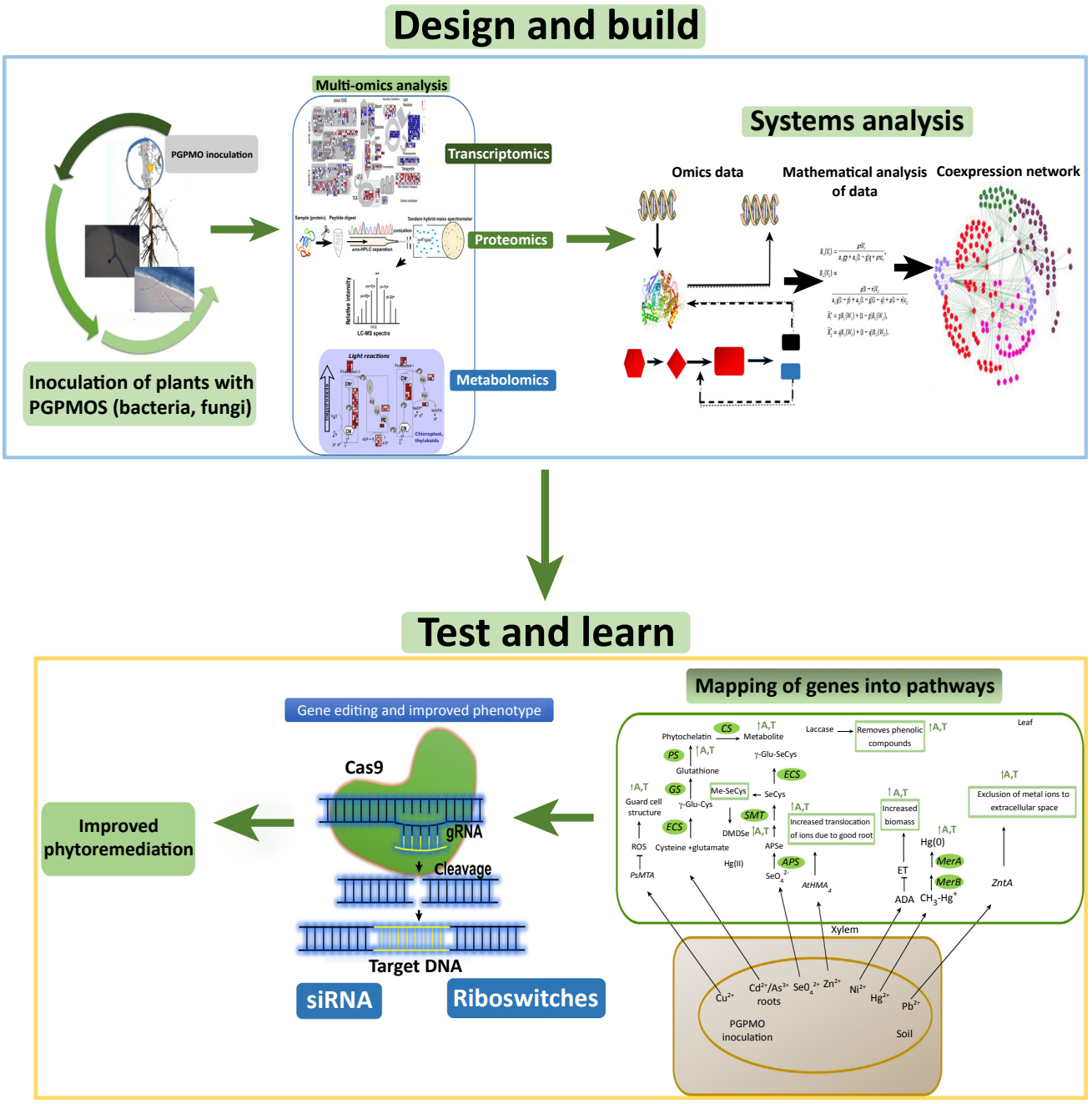

Figure 1. A Design–Build–Test–Learn (DBTL) Cycle Highlighting the Integration of Systems Biology and Gene Editing to Improve Phytoremediation. The design and build components validate gene expression (transcriptomics), proteins (proteomics), and metabolites (metabolomics); the learn component analyzes the large datasets generated by the design component through systems analysis. Information gathered by the design and build components can be employed for the identification of differential gene expression and putative candidate genes with phytoremediation functions in plants and plant growth-promoting microorganisms (PGPMOs) grown in a contaminated environment. The test and learn components confer with the implementation of gene editing tools to modify desired candidate genes to improve plant–PGPMO interactions for phytoremediation attributes. *APS*, ATP sulfurylase; *GS*, glutathione synthase; *ECS*, γ-glutamylcysteine synthase; *SMT*, selenocysteine methyltransferase; *CS*, cysteine synthase; *GS*, glutathione synthase; *PS*, phytochelatin synthase; *MerA*, mercuric ion reductase; *MerB*, organomercurial lyase; *AtHMA4*, P_{1B}-ATPase; *ZntA*, Zn(II)-translocating P-type ATPase; *ADA*, ACC deaminase; A, accumulation; T, tolerance; gRNA, guide RNA.

Table 1. A List of Genes Identified from Microbes and Their Hosts to Improve Phytoremediation

Gene	Host organism	Target	Function	Refs
Ohb (ortho-dechlorination gene)	*Pseudomonas aeruginosa*	*Comamonas testosteroni* strain VP44	Encodes enzymes to metabolize chlorobenzoic acids	[68]
FcbB (chlorobenzoatedehalogenase)	*Arthrobacter globiformis*, *Burkholderia* sp. strain DNT	*Pseudomonas fluorescens*	Degrades 2,4-dinitrotoluene	[69]
Toluene-*o*-monooxygenase	*Burkholderia cepacia* *Pseudomonas putida* F1	*P. fluorescens* *Deinococcus radiodurans*	Removes trichloroethylene (TCE); in highly irradiating environments, toluene, chlorobenzene, 3,4-dichloro-1-butene, and TCE are effectively oxidized	[70,71]
Polyphosphate kinase	*P. aeruginosa*	*P. aeruginosa*	Helps in cleaning up uranium	[72]
ArsB/ArsB/ArsC	*Staphylococcus aureus*	*Escherichia coli*	Confers resistance against arsenate by detoxification of arsenate by reduction	[73]
MerR (mercury resistance)	*Shigella flexneri*	*E. coli*	Confers resistance to Hg(II)	[74]
mtL (laccase gene)	*Myceliophthora thermophila*	*Saccharomyces cerevisiae*	Degrades lignin and polyaromatic hydrocarbons	[75]
Muconate and chloromuconate cycloisomerases	*P. putida*	*E. coli*	Catalyzed opening of ring for aromatic compounds	[76]
Cytochrome $P450_{CAM}$	*P. putida*	–	Oxidized hexane and 3-methylpentane	[77]
Toluene 4-monooxygenases, toluene 3-monooxygenase	*Ralstonia pickettii* PKO1 and *Pseudomonas mendocina* KR1	*B. cepacia* G4	Degraded the non-aromatic *N*-nitrosodimethylamine (NDMA), carcinogenic water contaminant for humans	[78]
bph operon (biphenyl)	*Burkholderia* sp. strain LB400	*P. fluorescens* F113	Improved the ability to degrade polychlorinated biphenyls (PCBs) and biphenyls	[79]
Chlorobenzenedioxygenase (CDO) gene under control of the *P. putida* Palk promoter	*P. putida*	*E. coli*	Overexpression of CDO gene catalyzed *cis*-dihydroxylation of aromatic compounds like benzonitrile	[80]
dszA/B/C (DBT monooxygenase)	*Rhodococcus erythropolis* *Chelatococcus* sp.	*Pseudomonas* strains –	Desulfurized DBT, also eliminates sulfur without hampering fuel content	[81]
Organophosphorus hydrolase (OPH)		*E. coli*	Efficiently degraded organophosphorus pesticide in a model reactor	[82]
vgb (bacterial hemoglobin gene)	*Vitreoscilla*	*E. coli*	Helped production of useful products and improved growth	[83]
lux gene fused within a naphthalene degradative pathway		*P. fluorescens* HK44	The recombinant microbes produce bioluminescence by degrading specific aromatic compounds like naphthalene	[84]
luc under Pu (*P. putida*) promoter and transcription activator	*P. putida*	*E. coli*	Estimated accumulated levels of toluene and toluene-like compounds in field water	[85]

Table 1. (continued)

Gene	Host organism	Target	Function	Refs
XylR (atrazine chlorohydrolase gene)		*E. coli*	Remediated soil contaminated with the herbicide atrazine	[86]
pnp operon (transforming *p*-nitrophenol into β-ketoadipate)	*Delftia acidovorans*	*P. putida*	Degraded an organophosphorus compound, paraoxon	[87]

expression of genes related to the degradation ability of polyaromatic hydrocarbons identified *Rhodococcus* as the most potent source of these genes. These examples paved the way for the use of **transcriptomics** technologies to identify and alter the PGPMO genes that are upregulated under contaminated environmental conditions and can be exploited to enhance phytoremediation processes.

Proteomics has emerged as a predominant analytical technique used to identify changes in protein expression in response to heavy metal stress in leaves of the **hyperaccumulator** plant *Phytolacca americana* [24,25]. Liu and colleagues [26] followed the same approach to understand the chronic effects of mercury (Hg^{2+}) in *Suaeda salsa*, identifying differential expression of proteins related to diverse metabolic pathways. Similarly, protein expression was studied in *Cupriavidus taiwanensis*, a Cd-tolerant bacterium, grown under Cd stress [27]. In short, a comparative proteomics analysis of plant–microbe interactions in a contaminated environment can identify key players for phytoremediation.

The potential of metabolomics technology in the investigation of microbial activities in a contaminated environment has been reviewed extensively. Metabolic and proteomic profiling of *Chelatococcus* sp. revealed the desulfurization pathway of dibenzothiophene (DBT) and its alkylated derivatives [28]. These studies show the potential application of omics technology in understanding metabolic pathways and gene–protein networks involved in bioremediation. Furthermore, they can be combined with other new analytical technologies like mathematical modeling or network biology to better understand the biological processes involved in the plant–PGPMO interaction.

The Plant–Microbe Metaorganism

A Combinatorial Omics Approach

Plant–microbe interaction in the rhizosphere is a complex process that involves both the symbiont and free-living soil microorganisms. In this context, one interesting, newly emerging concept is the **metaorganism** [29]. The idea of a metaorganism is a successful implementation of omics strategies that provides a clear understanding of the concomitant processes involved in the decontamination process mediated by the symbionts. It can detect the interdependence of various rhizospheric organisms and their hosts, which might go undetected if they are studied individually. Two primary questions governing a successful phytoremediation strategy are: (i) how plants trigger the assembly of beneficial microbes; and (ii) how the PGPMOs respond to stress signals. One logical way to answer these questions is to use dual transcriptomics across different conditions that will highlight ways to maximize the benefits of phytoremediation from the metaorganism, consequently allowing the translation of omics knowledge to useful technologies for the future. Various omics approaches used to generate enormous amounts of data on plant–microbe interactions thereby constitute the build section of the DBTL cycle, but they need to be combined into one using a systems biology approach

and mathematical modeling to easily translate knowledge from the laboratory to a successful implementation strategy and hence provide the foreground for testing the experimental approach. The identification of candidate genes from systems analysis leads to the important goal of learning the outcomes of the design, which can be achieved by gene editing strategies and hence is a step forward towards the development of new strategies for the future.

Integration of Large Omics Datasets

Systems biology can help in studying the multiple levels of interactions occurring within a living cell, plant, or microbial community and their relationships to the various physiological and biochemical processes in the ecosystem. Understanding the different datasets generated from omics requires the utilization of various software and tools for data management, network construction, and, finally, model analysis. The recent emergence of network biology as a principal tool for systems analysis has enabled the integration of multi-omics data into one dataset by mathematical analysis of the relationships between several interconnected objects in a biological system [30]. Network analysis results in an interactome model that can be used to identify the molecular mechanism or putative candidate genes. Several systems biology tools are routinely used to stud plant–microbe interactions (Box 2). Recently, a new computational tool called Mergeomics [31,32] was developed to identify disease-associated processes with the enormous datasets that were generated from omics studies of plant–microbe interactions. Researchers can use this pipeline to identify key regulators and other important components involved in a phytoremediation process. Some of the genes mentioned in Table 1 and Figure 1 are candidates for gene manipulation to study the effectiveness of phytoremediation.

Implementing the Knowledge Gained from Systems Analysis

Manipulating the Host and the Microorganisms: Transgenic Technology in Phytoremediation

A successful phytoremediation strategy relies on multiple factors, including plant genotypes and the interaction between the plant and its environment. Genetic engineering of plants by overexpression of metal ligands, transporters like *PvACR3*, transcription factors like *AtPHR1*, or

Box 2. Bioinformatics and Web-Based Tools for Systems Analysis

Systemic analysis of omics data has been made easier by the advent of web-based or otherwise *in silico* analysis pipelines.

Constraint-based modeling (CBM) is a modeling system that combines genomic, biochemical, and genetic information into a mathematical structure that mechanically explains the physiology of metabolism.

Omics focuses on characterizing and quantifying biological molecules to give an idea of the structure, function, and dynamics of an organism. Example omics disciplines are genomics, transcriptomics, metabolomics, and proteomics.

Flux balance analysis (FBA) is a mathematics-based modeling system that can simulate metabolic pathways when metabolic networks are reconstructed on a genome-wide scale.

KeyPathwayMinerWeb (https://keypathwayminer.compbio.sdu.dk/keypathwayminer/) is an online platform that enables pathway enrichment analysis (*de novo*) directly in the browser.

Omics (https://pypi.python.org/pypi/omics) is a Python-based data package aimed at integrative genomics analysis.

Omictools (http://omictools.com/) is a metadatabase of >4400 tools that is primarily focused on microarray, NGS, PCR, mass spectrometry (MS), and NMR techniques [60].

Optknock helps to identify genes that are overproducers in a biochemical pathway that can be deleted.

enzymes involved in sulfur metabolism can lead to successful phytoextraction [33–36] (Figure 1). The interaction of plants with PGPMOs has been successfully used to clean up soils contaminated with uranium (U), Pb), and zinc (Zn) [37,38] (Table 1). This detailed information about genes involved in bioremediation can be exploited in the future to modify PGPMOs to improve phytoremediation in polluted environments.

siRNAs: An Alternative Method for Improving Phytoremediation

Small RNAs are noncoding RNA molecules that are involved in the regulation of gene expression, thereby exerting control over several cellular processes, like protection against pathogen attack, and physiological processes including response to heavy metal stresses. RNA silencing in plants is mediated by siRNAs and miRNAs. The formation of RNA-induced silencing complexes (RISCs) in response to heavy metal stress provides protection by: (i) forming complexes with the metals; (ii) post-transcriptional processing of the target RNA; or (iii) transcriptionally controlled methylation of the target DNA. Thus, post-transcriptional regulation of gene expression can be successfully implemented to improve the plant–PGPMO interaction for better phytoremediation of contaminated soil.

Riboswitches and Ribozymes

Riboswitches are RNA elements that regulate the expression of mRNA by binding to a ligand that binds to the aptamer domain. Some advantages of riboswitch technology are that it does not require additional proteins or a heterologous system and that it allows direct administration of ligands [39]. For example, the motility of *E. coli* can be controlled by the genetic engineering of riboswitches into *cheZ*, the gene that controls chemotaxis. Moreover, some synthetic riboswitches have been developed to control gene expression in bacteria conditionally [40]. Ribozymes are catalytic RNAs that interact with ligands that are then perceived by the riboswitches, leading to the regulation of gene expression by controlling transcription, the stability of RNA, translation, or splicing [39]. Ribozymes can have a promoting or inhibitory effect on gene expression. One example is the *glmS* ribozyme coupled to GlcN6P, recently identified in *Bacillus anthracis*, which controls the expression of glucosamine 6-phosphate (GlcN6P) synthase by binding to the metabolite GlcN6P [41]. By identifying RNA sequences in yeast, it is now possible to introduce an activator or repressor in the promoter region of a selection marker, or an aptamer recently found to induce transcription in *E. coli*, by replacing the helix–turn–helix motif from the repressor protein *TetR* [40]. These techniques could also be used to modify PGPMOs or the host plant to improve phytoremediation efficiency (Box 3).

Gene Editing: Customizing Plant–Microbe Interactions to Improve Phytoremediation

Gene editing has already found applications in a variety of fields in making customized changes in desired locations in DNA. However, gene editing differs from traditional gene manipulation techniques that do not necessarily incorporate foreign DNA [42,43]. Recently, **gene editing tools** like CRISPR–Cas9 and CRISPR–Cpf1 have shown the potential to improve agronomics and improve phytoremediation efficiency by improving plant–PGPMO interactions (Box 4). CRISPR–Cas9 technology has been successfully implemented to improve crops for

Box 3. Role of Riboswitches in Phytoremediation

The discovery of riboswitches over a decade ago has given scientists an alternative possibility to explore the functions of the genes in a signaling network within cells. Both natural and synthetic riboswitches can be used to regulate gene expression in a ligand-dependent manner. Riboswitches are becoming an increasingly powerful tool for biologists in bioremediation studies by providing an easy understanding of the mechanisms of gene interaction in a regulatory pathway. The presence of riboswitches in bacteria that enable binding to specific metabolites without the need for any additional protein have been used for removal of heavy metals [61–63].

Box 4. Gene Editing Tools

Some of the genes mentioned in Table 1 and Figure 1, and new genes identified through systems analysis, are potential candidates for improving the efficiency of phytoremediation by generating transgenic plants and microbes using the gene editing techniques mentioned below.

CRISPR–Cas9 is a newer gene editing technology that has been widely used in plants for crop improvement [64,65]. Cas9 is a RNA-guided DNA endonuclease that is targeted to specific sequences in the genome by genetically engineering a guide RNA that forms a complex with Cas9.

CRMAGE is a highly efficient and new technology for gene editing in *Escherichia coli* that combines CRISPR–Cas9 and Lambda (λ) Red recombineering with the MAGE technique. However, the technique is limited by the availability of the recombinase system in the host [66].

Multiplex genome editing by natural transformation (MuGENT) promotes the integration of mutations with high efficiency over a short time in the genome and can also generate a complex population of mutants that can be used for metabolic engineering [67].

Recombineering is a modified CRISPR–Cas9 that uses homologous recombination mediated by lambda (λ) recombinase coupled to CRISPR–Cas9. It is becoming an increasingly popular method for gene editing of *E. coli* or other microbes due to its increased efficiency of mutagenesis and robustness.

RNA-guided endonucleases (RGENs) are ribonucleoproteins comprising Cas9 and guide RNA derived from *Streptococcus pyogenes*. They play a pivotal role in targeted modification of the genome but are limited by undesirable translocation of the chromosome arising from mutations and DNA cleavages that are off target [67].

Transcription activator-like effector nucleases (TALENs) use nonspecific exonucleases fused to a DNA-binding domain and can be easily engineered to create changes in any specific portion of the DNA sequence.

Zinc-finger nucleases (ZFNs) are a class of genetically engineered DNA-binding proteins that enable genome editing at targeted sites by initiating double-strand breaks in the DNA.

commercial use as well as to improve yield. For instance, this gene editing technique has been used to improve the oil composition of *Camelina sativa* seeds, which has enriched fatty acid [44], and similarly to modify the *SP5G* (self-pruning) gene in tomato, which resulted in a bushy, early-yielding phenotype [45]. Additionally, gene editing has been used to identify new alleles by mutagenizing *OST2* [46] and to develop resistance against powdery mildew disease in wheat and against bacterial blight disease in rice [47,48]. There are many examples of successful manipulation of metabolic pathways using CRISPR–Cas9 tools, where mutations in the rice ERF transcription factor *OsERF922* led to enhanced resistance against *Magnaporthe oryzae* and mutations in the *ALS2* (acetolactate synthase) gene of maize resulted in tolerance to chlorsulfuron [49–51]. Other applications of these technologies include the alteration of major target genes in microbes, like quorum sensing and plant–microbe signaling pathways, to optimize symbiotic and beneficial interactions [28,52].

Existing knowledge and information suggest that gene editing tools have not yet been used to modify PGMPOs for phytoremediation, but gene editing tools could be used to knock in, overexpress, or delete a gene of interest to improve phytoremediation. As shown in Table 1, multiple genes in microbes were discovered to have phytoremediation attributes, like genes that encode enzymes involved in the degradation of contaminants such as chlorobenzoic acid, 2,4-dinitrootoluene, trichloroethylene, and arsenate. These genes have shown great potential to reduce soil contaminants and can be overexpressed by employing gene editing tools in PGPMOs. Similarly, rhizobacteria that produce indole-3-acetic acid (IAA) enable plants to resist metal stress and improve nitrogen fixation as well. By utilizing gene editing tools, these

rhizobacteria can be customized to improve the IAA threshold in the rhizosphere. An emerging arena of work shows that PGPMOs such as *Bradyrhizobium elkanii*, *Sinorhizobium* sp. and *Rhizobium* spp. have abilities to overcome the **ethylene effect** [53] on root growth inhibition by producing rhizobitoxine or ACC deaminase [54–56]. These genes could be potential targets for gene editing to improve metal remediation and plant growth simultaneously. Furthermore, a revolutionary demonstration of CRISPR machinery to uncover features of spacer acquisition, which is required to adapt the CRISPR–Cas procedure, lays the ground for an intracellular digital recording device [57]. This system suggests the eventual possibility of tracing long histories of the lineage, adaptation, and molecular experiences of bacterial cells. These innovative developments in technology can be utilized to develop custom-made PGPMOs to improve phytoremediation. In short, gene editing tools like the Cas9/sgRNA system can be effectively improved for the deletion or insertion of target genes to customize desired improvements in plants and PGPMOs for phytoremediation [58].

Concluding Remarks and Future Perspectives

The symbiotic association between plants and PGPMOs has been shown to play a pivotal role in phytoremediation as well as in plant health improvement. Despite the beneficial aspects of this association, a tradeoff still exists between the participants, so more insightful human discovery remains necessary to optimize the plant–microbe interaction. A multidimensional phytoremediation strategy involving plant–microbiome interactions requires a well-orchestrated combination of various omics technologies along with skillful implementation of gene editing and other transgenic approaches to introduce foreign genes (Figure 1). However, for PGPMOs to become a widely accepted phytoremediation methodology in the future, a comprehensive understanding of plant–microbe interactions is essential, along with a thorough knowledge of microbial functions that play pivotal roles in improving phytoremediation efficiency. A DBTL cycle as suggested by Campbell and colleagues [59] is an ideal and timely approach to integrate the large omics dataset into a systematic analysis enabling the identification of candidate genes that can be manipulated by gene editing techniques to learn about and fully characterize their functions (Figure 1).

Therefore, in our opinion it will be beneficial to combine and employ the current knowledge of systems biology with gene editing tools to further establish and enhance phytoremediation through plants and microbe interactions. However, the practical and successful implementation of gene editing techniques to engineer microorganisms remains uncertain due to dangers to the environment and society and the ability of these organisms to survive in a natural environment. Furthermore, there are ethical concerns related to the containment of genetically modified organisms and their impact on Earth's ecosystem (see Outstanding Questions). However, the vivacity of organisms and their genetic constitution clearly indicate exciting prospects for future research and exploration.

Outstanding Questions

Can modified PGPMOs still interact with plants in native environments, compared with controlled environments?

How do we limit PGPMOs to a niche instead of them spreading in the ecosystem?

What are the unforeseen implications of releasing modified PGPMOs into the environment?

Can we use DNA-free gene editing technologies to improve the phytoremediation capacity of PGPMOs and overcome social concerns?

Can we exploit riboswitch technology to make plant–PGPMO interactions favorable under shifting environmental conditions?

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